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Identification of *Campylobacter jejuni* Genes Involved in Its Interaction with Epithelial Cells▽†

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***Campylobacter jejuni* is the leading cause of infectious gastroenteritis in industrialized nations. Its ability to enter and survive within nonphagocytic cells is thought to be very important for pathogenesis. However, little is known about the *C. jejuni* determinants that mediate these processes. Through an extensive transposon mutagenesis screen, we have identified several loci that are required for *C. jejuni* efficient entry and survival within epithelial cells. Among these loci, insertional mutations in *aspA*, *aspB*, and *sodB* resulted in drastic reduction in *C. jejuni* entry and/or survival within host cells and a severe defect in colonization in an animal model. The implications of these findings for the understanding of *C. jejuni*-host cell interactions are discussed.**

Campylobacter jejuni is one of the most important causes of food-borne illness in industrialized nations and diarrhea in children in developing countries (45, 67). Despite its importance as a pathogen, its virulence mechanisms are just beginning to be understood. The ability of *C. jejuni* to enter nonphagocytic cells is thought to be very important for its pathogenesis (77). Correlation between the cultured-cell invasiveness of *Campylobacter* strains and the severity of the disease outcome has been reported (10, 11, 33, 47), and studies have visualized *C. jejuni* inside intestinal epithelial cells during human infections (68). *C. jejuni* can enter and survive within a variety of cultured cell lines (37, 49, 71, 73). Studies have revealed unique aspects in the cell biology of *C. jejuni* entry. For example, it has been shown that *C. jejuni* entry does not require an intact actin cytoskeleton although it requires an intact microtubular network (49). Other studies have implicated Rho-family GTPases in *C. jejuni* entry (39). However, little is known about the bacterial determinants specifically involved in mediating entry and intracellular survival. Although several studies have identified *C. jejuni* mutants exhibiting various degrees of deficiency in their ability to enter cultured cells (1, 2, 5, 13, 17, 25, 27, 29–31, 52, 63), there is no evidence indicating that the identified gene products directly mediate the entry process. Nonmotile mutants exhibit a drastic decrease in their ability to invade cultured cells (17, 70, 75). However, it is unclear whether the flagellar structure is directly involved in triggering bacterial internalization or whether the severe entry defect of nonmotile mutants indicates that motility *per se* is required for entry. A mutation in the *pflA* gene, which results in paralyzed flagella, has been shown to be defective for entry (75), suggesting that motility and not the flagellar structure itself is required for entry. However, the actual role of PflA is unknown, and therefore it is still possible that the phenotype of the *pflA* mutation may be due to functions other than its putative role in motility. We have carried out a mutagenesis screen to identify *C. jejuni* genes that are required for entry and/or survival within host cells. Among the identified loci, insertional mutations in *aspA*, *aspB*, and *sodB* resulted in a drastic reduction in *C. jejuni* entry and/or survival within host cells and a severe colonization defect in an animal model. Although our studies did not provide evidence for a direct involvement of these loci in the cell entry process, these findings highlight the importance of *C. jejuni* basic metabolism in its ability to interact with host cells.

MATERIALS AND METHODS

Bacterial strains, cell lines, and culture conditions. The complete list of strains and plasmids used in this study is shown in Table 1. The expression vector pKETH-8c that carries the Tn*552* transposase with an N-terminal His tag (59) was kindly provided by Nigel D. F. Grindley. The *C. jejuni* derivative of the Tn*552* transposon (Tn*552kan-Campy*) has been described previously (7). The *Escherichia coli* BL21(DE3) strain (64) (Invitrogen) was used for the expression and purification of the TnpA transposase using standard affinity chromatography techniques. *E. coli* XL1-Blue (Stratagene) or DH5α (Bethesda Research Laboratories) was used as a host strain for recombinant DNA experiments and other genetic manipulations. For *E. coli*, antibiotics were used at the following concentrations as needed: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and chloramphenicol, 30 μg ml^{-1}. All *E. coli* strains were stored at −80°C in Luria-Bertani (LB) medium containing 20% glycerol.

The *C. jejuni* 81-176 wild-type strain used in this study has been described previously (4, 24, 38) and was a generous gift from Patricia Guerry. Routinely, *C. jejuni* was grown on brucella broth agar or on blood agar plates (Trypticase soy agar supplemented with 5% defibrinated horse blood [Beckton & Dickinson]) at 37°C in an incubator equilibrated to a 10% CO_2 atmosphere or under low-oxygen conditions (GasPak Plus; BD-Diagnostic Systems, NJ). The *C. jejuni* transformants were selected on plates supplemented with 50 μg ml^{-1} kanamycin and 7.5 μg ml^{-1} chloramphenicol, as indicated below. For liquid cultures *C. jejuni* strains were grown in brain heart infusion (BHI) medium with no antibiotics added. For the determination of growth curves, *C. jejuni* cultures were adjusted to an optical density at 600 nm (OD_{600}) of about 0.1 and placed on a rotating wheel (50 rpm) at 10% CO_2 for 14 h. For large-volume cultures, *C. jejuni* strains were incubated under the same conditions using an orbital shaker at 200 rpm. All *C. jejuni* strains were stored at −80°C in BHI broth containing 30% to 50% glycerol. In all cases,

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype (ORF) or description	Reference and/or source
Strains		
E. coli		
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	64; Invitrogen
DH5α	λ$^-$ Φ80d*lacZ*DM15 D(*lacZYA-argF*)*U169 recA1 endA hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F− *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)	Stratagene
C. jejuni		
81-176	Wild type; human isolate	4, 38; gift of Patricia Guerry
CB1	*flaA*::*aphA3* (*cjj81176_1339*::*aphA3)*	Laboratory strain collection
CB18	*motA*::*aphA3* (*cjj81176_0359*::*aphA3*)	This study
CB21	complemented *aspA* mutant; *aspA*::*aphA3* (*cj81176_1539*::*cat aspA*)	This study
CB22	complemented *aspB* mutant; *aspB*::*aphA3* (*cj81176_1539*::*cat aspB*)	This study
CB25	*peb1A*::*aphA3* (*cjj81176_0928*::*aphA3*)	This study
CB26	*ciaB*::*aphA3* (*cjj81176_0921*::*aphA3*)	This study
CB30	*sodB*::*aphA3* (*cjj81176_0205*::*aphA3*)	This study
CB43	*cj0977*::*aphA3*	This study
CB60	*cjj81176_0996*::*aphA3*	This study
CB61	*cjj81176_0708*::*aphA3*	This study
CB62	*jlpA*::*aphA* (*cjj81176_1002*::*aphA3)*	This study
Plasmids		
pKETH-8c	His-tagged p480 (TnpA) transposase in pET expression vector; Km^r	59; gift of Nigel D. F. Grindley
pTG426	Source of Tn*552cat*; *cat cassette flanked by 48-bp Tn552 terminal inverted repeats in pUC19*	18; gift of Nigel D. F. Grindley
pSB1699	Source of Tn*552kan-Campy;* Tn*552aphA3* in SpeI of pRY112	7
pRY109	Source of the chloramphenicol acetyltransferase gene (*cat* cassette)	74
pBluescriptII SK(+/−)	Phagemid cloning vector, f1 origin in plus orientation; *lacPOZ′*; Sac → Kpn polylinker orientation; Amp^r	Stratagene
pSB3021	*C. jejuni* complementation vector; pGK2003 *cjj81176_1539*::*cat*	72
pSB2996	*cjj81176_1539*::*cat sodB* in pSB3021	This study
pSB3000	*motA*::*aphA3* in pBluescript II SK (+/−)	This study
pSB3001	*peb1A*::*aphA3* in pBluescript II SK (+/−)	This study
pSB3002	*cj0977*::*aphA3* in pBluescript II SK (+/−) (*cj81176_0996*::*aphA3*)	This study

cultures were inoculated by swabbing bacteria from plates incubated for 12 to 24 h (to avoid experimental discrepancies due to phase variation).

COS-7 (African green monkey kidney fibroblast-like cell line) or T84 (human colon carcinoma cell line) cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cell lines were kept under a 5% CO_2 atmosphere. T84 cells were used in gentamicin protection assays because these cells provide better support for *C. jejuni* entry. COS-7 cells were used in all experiments involving microscopy and fluorescence-activated cell sorter (FACS) analysis since they are more amenable than T84 cells for these types of assays and support robust *C. jejuni* entry.

DNA manipulation and *C. jejuni* strain construction. The complete list of primers used in this study is shown in Table S1 in the supplemental material. DNA manipulations were done according to standard laboratory protocols described elsewhere (60). For isolation of genomic and plasmid DNA, a DNeasy tissue kit and a QIAprep spin miniprep kit (Qiagen) were used.

The *C. jejuni* 81-176 *peb1A*, *cjj81176_0996* (homolog of Cj0977 in the NCTC 11168 reference strain), *motA* (*cjj81176_0359*), *jlpA*, and *ciaB* mutant strains were constructed by PCR amplification of the flanking regions of these open reading frames (ORFs) with specific primers (see Table S1 in the supplemental material) and cloning of a kanamycin resistance cassette (*aphA3*) between the amplified flanking regions. The resulting plasmids (built on a pBluescript II SK backbone) were used to move the mutated alleles into the chromosome of *C. jejuni* 81-176 by natural transformation and allelic recombination.

The mutated strains of *C. jejuni* were complemented by introducing a wild-type copy of the different alleles (i.e., *sodB*, *aspA*, and *aspB*), as previously described (72). Briefly, complementation was carried out by integrating the complementing gene within the *hsdM* locus and driving its constitutive coexpression with a chloramphenicol resistance gene.

Construction of *C. jejuni* transposon mutant library. An *in vitro* transposon mutagenesis system was used to generate insertion mutants of *C. jejuni* as previously described (7). Briefly, 20-μl reaction mixtures containing ~500 ng of chromosomal DNA, 100 to 150 ng of Tn*552kan-Campy*, and 100 ng of TnpA in 20 mM HEPES (pH 7.0), 50% (wt/vol) glycerol, 125 mM NaCl, and 10 mM $MgSO_4$ were incubated at 37°C for 60 min and then dialyzed against water using 0.025-μm-pore-size nitrocellulose membrane discs (Fisher). *C. jejuni* electrocompetent cells were prepared as previously described (20) and were transformed directly with a portion of the dialyzed transposition reaction mixture. The mixture of competent cells and DNA was incubated on an antibiotic-free blood agar plate for 5 to 7 h and then resuspended in 400 μl of BHI medium and plated out on brucella broth plates containing kanamycin (50 μg ml^{-1}). The insertion sites of the different transposon mutants were determined by DNA sequencing using *aphA5′* outward and *aphA3′* outward primers (see Table S1 in the supplemental material).

Screening of *C. jejuni* transposon mutants for their ability to enter cultured cells. The protocol used to screen the *C. jejuni* mutant library for mutants unable to enter cultured cells is outlined in Fig. S1 in the supplemental material. To evaluate invasion abilities of a large number of Tn*552kan-Campy* transformants, a modified gentamicin protection assay was used to address the following issues: (i) the screen had to allow handling a large number of mutants at once; (ii) no liquid culture and no optimization of optical density could be utilized for logistical reasons; (iii) the growth conditions had to allow the wild-type *C. jejuni* strain to retain its invasiveness; and (iv) all steps of the screen had to be reliable and easy to monitor. Individual 2-day-old Tn*552kan-Campy* transposon mutant colonies were transferred to the center of soft-agar wells (0.8%, wt/vol, agar in brucella broth) of 24-well polystyrene plates and incubated at 37°C under 10% CO_2 for 24 h. Growth on a soft agar allowed us to assess motility of each *C. jejuni* mutant, and only motile clones were considered for further analysis since non-

motile mutants are noninvasive (see below) (14, 17, 70, 75). After the 24 h of growth at 37°C in a 10% CO_2 atmosphere, 500 μl of brucella broth medium was added to each well, and cells were incubated for an additional 12 to 14 h (biphasic growth). Bacterial cells were lifted off the plate into the overlaid medium by gentle mixing and used as an inoculum in the invasion assay.

T84 cells were seeded to ~70% confluence in 24-well plates (seeded at about 10^5 cells per well) and washed three times with Hank's balanced salt solution (HBSS). Fifteen microliters of bacterial suspension was added with 500 μl of HBSS to each well, and plates were spun down at 200 × *g* for 5 min to enhance bacterium-host cell contact, followed by incubation for 2 h at 37°C in 5% CO_2. The motility of each mutant was examined under a light microscope (Nikon TMS 0.3A; ×40 objective with a 1.4 numerical aperture [NA]) at 1 h postinfection, and the approximate percentage of the motile versus nonmotile population of bacteria was recorded. Infected monolayers were washed three times with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) containing 0.5 g/liter gelatin (BSG), and 500 μl of prewarmed DMEM containing gentamicin at 150 μg ml^{-1} was added for 3 h to kill extracellular bacteria. The intestinal cells were washed again and lysed with 500 μl of 0.1% sodium deoxycholate in PBS to release intracellular bacteria. The invasion ability of the assayed mutants was evaluated by overlaying 25 μl of cell/bacteria lysate dilution (1:200 in brucella broth medium) onto blood agar plates (allowing the evaluation of four mutants per plate), followed by a 3-day incubation. We assumed that mutants had an invasion defect if the number of recovered bacteria was ~50% or less of that of the wild-type strain. Invasion-defective mutant candidates from the initial screen were reevaluated later in a standardized bacterial invasion assay as described below.

Standardized bacterial invasion assay. The ability of *C. jejuni* to invade cultured intestinal epithelial cells was evaluated by the gentamicin protection assay using a *C. jejuni flaA* knockout strain (nonmotile and noninvasive) as a negative control. Briefly, T84 cells were split to ~70% confluence in 24-well plates (seeded at ~10^5 cells per well) 1 day prior to the experiment. To prepare bacterial inocula, *C. jejuni* strains were grown in BHI medium to an early logarithmic phase (OD_{600} of ~0.3 to 0.5) and then adjusted to an OD_{600} of ~0.1 in HBSS medium. Serial dilutions of the inoculum were plated out onto blood agar plates to determine the number of bacteria. On the day of the experiment, cell monolayers were washed three times with 500 μl of HBSS and 10 μl of bacterial cells (~10^6 bacteria; at a multiplicity of infection [MOI] of 10) was added to each well. Plates were spun down at 200 × *g* for 5 min to enhance bacterium-host cell contact and incubated for 2 h at 37°C under 5% CO_2. Following the incubation, infected monolayers were washed three times with BSG, and prewarmed DMEM containing gentamicin (150 μg ml^{-1}) was added for 3 h to kill extracellular bacteria. Cells were washed again and lysed with 0.1% sodium deoxycholate (Sigma) in PBS to release intracellular bacteria. Invasion ability was expressed as a percentage of the inoculum that survived gentamicin treatment. Intracellular bacteria were enumerated by plating serial dilutions on blood agar plates. Each assay was conducted in triplicate and was independently repeated at least three times.

Intracellular survival assay. The ability of *C. jejuni* to survive within cultured intestinal epithelial cells was evaluated as described earlier (71) with some modifications. T84 cells were seeded at ~10^5 cells per well and infected at an MOI of 20. After a 2-h infection cell monolayers were washed three times with BSG, and prewarmed DMEM containing gentamicin (150 μg ml^{-1}) was added to each well for 3 h to kill extracellular bacteria. Cells were lysed, and bacteria were plated under 10% CO_2. Alternatively, infected cells were washed and incubated with DMEM containing gentamicin (20 μg ml^{-1}) for 24 h. The low concentration of antibiotic does not affect the internalized bacteria over this time period (71), and the antibiotic was left in the medium to prevent growth of extracellular bacteria that escaped the previous treatment with gentamicin. On the next day, cells were washed three times with BSG and lysed, and released intracellular bacteria were plated and incubated under anaerobic (GasPak Plus) conditions. The ability to survive inside intestinal cells was expressed as a percentage of bacteria recovered after 24 h under anaerobic conditions (second plate) relative to the number of bacteria recovered after 5 h under microaerophilic conditions (first plate), which was set as 100%.

Swarm plate assay. The optical density of the bacterial culture to be tested was adjusted to an OD_{600} of ~0.1, and 2 μl was spotted onto soft agar (0.5%, wt/vol). Plates were incubated for ~24 h at 37°C, and then the swarming diameter of the tested strain was compared to that of the wild-type and the nonmotile, *flaA* (or *motA*), strains.

Immunofluorescence microscopy. Enumeration of intracellular and extracellular bacteria was carried out using a staining protocol capable of distinguishing internalized versus extracellular bacteria, as previously described (12). Briefly, COS-7 cells were grown on poly-L-lysine-treated 12-mm round glass coverslips to ~70% confluence and then infected with different strains of *C. jejuni* at an MOI of 50 in HBSS for 2 h. Cells were briefly washed with PBS, fixed in 2% paraformaldehyde solution for 15 min at room temperature, and incubated (blocked) in 5% suspension of nonfat dry milk (Carnation) in PBS for 30 min. Fixed cells were incubated with rabbit anti-*C. jejuni* 81-176 serum in PBS–5% (wt/vol) milk for 30 min. Cells were washed three times with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000; Molecular Probes) for 30 min. The coverslips were washed with PBS and permeabilized with PBS–0.05% (wt/vol) saponin for 15 min. Infected cell monolayers were incubated again with serum against the *C. jejuni* 81-176 strain, washed three times with PBS, and covered with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:1,000; Molecular Probes) in PBS-saponin–5% milk for another 30 min to stain the intracellular bacteria. Cell nuclei were distinguished by DAPI (4′, 6-diamidino-2-phenylindole) staining. The coverslips were mounted onto glass slides with ProLong Gold antifade reagent (Molecular Probes), and the numbers of invading bacteria per cell were quantified under a fluorescence microscope (Nikon Diaphot 300) with a ×100 (1.4 NA) oil immersion objective. Results represent the mean ± standard deviation (SD) of three separate experiments with at least 100 cells counted in each experiment.

Sensitivity of *C. jejuni* strains to sodium deoxycholate. To determine the sensitivity of sodium deoxycholate, *C. jejuni* strains were adjusted to a concentration of about 10^8 bacteria per ml in HBSS. A total of 100 μl of bacterial cell suspension was transferred to microcentrifuge tubes containing 900 μl of either 0.1% sodium deoxycholate or HBSS solution. Samples were left at room temperature for 20 min and then subjected to a series of dilutions in HBSS and plated out onto blood agar plates. The numbers of CFU were enumerated, and the percentage of bacteria that survived the detergent treatment was compared between strains using the *C. jejuni* wild-type strain as a reference. Early-logarithmic-phase bacterial cultures were used in this test. The incubation time in 0.1% sodium deoxycholate exceeded the time needed to lyse COS-7 (up to 1 min) or T84 (up to 7 min) cells during the standardized invasion assay.

Mouse infections. Animal infection studies were carried out using *myd88*$^{-/-}$ *nramp1*$^{-/-}$ male mice as previously described (72). Briefly, *myd88*$^{-/-}$ *nramp1*$^{-/-}$ male age-matched (6 to 10 weeks old) mice were infected intraperitoneally (i.p.) with 10^7 CFU of different *C. jejuni* strains. *C. jejuni* strains to be tested were restreaked from 1-day-old frozen stock the night before the experiment and then grown in BHI medium to an OD_{600} of ~0.4 to 0.6. The colonization levels of the different *C. jejuni* strains were monitored by enumerating the number of CFU in the feces of inoculated animals. At the time points indicated in Fig. 5 and 6, feces were collected into BHI broth, weighed, and plated on blood agar plates containing *Campylobacter*-selective supplements (Oxoid SR0167E) to determine the number of CFU per gram of feces. To differentiate between *C. jejuni* strains, the antibiotic kanamycin (50 μg ml^{-1}) or chloramphenicol (5 μg ml^{-1}) was added to selective medium when appropriate. At the end of the experiment mice were sacrificed, and their organs were aseptically removed and homogenized in HBSS. The bacterial loads in the intestine, liver, and spleen of infected animals were enumerated by plating 10-fold serial dilutions on selective plates, as described above. Statistical analysis of the results was carried out with a Wilcoxon matched-pair signed-rank test.

Enumeration of *C. jejuni* loads in host cells by flow cytometry. To enumerate bacteria by flow cytometry, the protocol described by Watson et al. was followed (72). Briefly, COS-7 cells were seeded at a density of 1 × 10^5 cells per well on a 24-well dish and infected at an MOI of 50. Following a 1-h incubation at 37°C and 5% CO_2, the cells were washed with HBSS, and DMEM containing 10% FBS and gentamicin (150 μg ml^{-1}) was added to each well. Cells were washed again and lysed at the time points indicated in Fig. 3 in 500 μl of 0.1% sodium deoxycholate in PBS. The cell lysates were collected and subjected to a low-speed spin (1,000 rpm) for 1 min to remove large cell debris. Supernatants were collected, and intracellular bacteria were recovered by a 2-min high-speed spin (10,000 rpm). The isolated bacterial pellet was resuspended in 500 μl of filter-sterilized staining buffer (PBS containing 1 mM EDTA and 0.01% Tween). Bacteria were then stained with the reagents of a cell viability kit (BD Biosciences, San Jose, CA), which distinguishes live and dead cells by using a thiazole orange (TO) solution, which stains all bacteria, and propidium iodide (PI), which stains only dead bacteria. The TO and PI solutions were added to final concentrations of 53 nM and 11 μM, respectively, in accordance with the manufacturer's instructions. After 5 min of staining, bacteria were pelleted, washed once in PBS, resuspended in 1 ml of PBS, and analyzed by flow cytometry. The absolute count of live and dead bacteria was carried out by the addition of 50 μl of a liquid suspension of a known number of fluorescent beads (supplied with the kit; BD Biosciences, San Jose, CA) following the manufacturer's instructions. Samples were analyzed on a FACSCalibur flow cytometer. TO fluo-

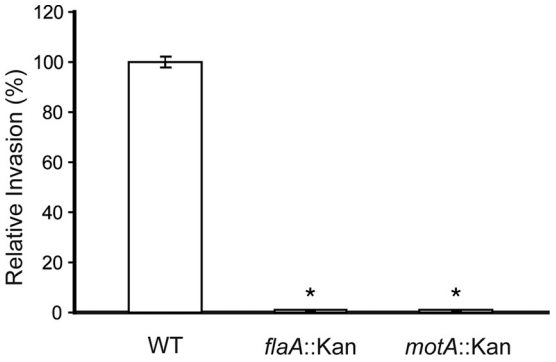

FIG. 1. Motility but not the flagellar structure is essential for *C. jejuni* entry into cultured cells. T84 cells were infected with the wild-type *C. jejuni* 81-176 strain (WT) or its isogenic *flaA*::*kan* or *motA*::*kan* mutant at an MOI of 10 for 2 h, followed by a 3-h incubation in the presence of gentamicin. A comparison of the levels of invasion is shown as the percentage of bacteria that survived treatment with gentamicin relative to that of the wild-type strain, which was set at 100%. The error bars represent the standard deviation of three independent determinations. *, values were statistically significantly different ($P = 0.0001$, Student t test) from those of the wild type.

resces primarily in FL1 and FL2, and PI fluoresces primarily in the FL3 channel. A side scatter (SSC) threshold was used, and cells and beads were gated using scatter and FL2, which detects the TO fluorescence and therefore the total bacterial population. In order to best discriminate between live and dead bacteria, a plot of FL1 versus FL3 was used, and live and dead populations were gated within this plot (dead cells, FL3 positive; live cells, FL1 positive). To determine the bacterial concentration, the following equation was used: number of events in the cell region/number of events in the bead region × number beads/test/test volume × dilution factor = concentration of the cell population. A plot was generated after this equation was used to calculate the number of viable bacteria (in triplicate wells) at each time point.

RESULTS

***C. jejuni* 81-176 *motA* mutant is deficient for bacterial entry into cultured epithelial cells.** Although flagellar mutants are drastically reduced in their ability to enter cultured epithelial cells, it is still unclear whether the invasion defect is due to the lack of motility, a direct role of flagellin in the entry process, or a potential role of the flagellar apparatus as an export machine for a putative invasion factor (19). Therefore, we compared the invasive ability of the wild-type *C. jejuni* 81-176 with that of the nonmotile derivatives *flaA* and *motA*. The *flaA* mutant lacks flagellin (the main filament subunit), whereas the *motA* mutant makes a complete flagellar structure but is nonmotile because of its inability to rotate the flagella. The *flaA* and *motA* mutants are predicted to be fully competent for secretion through the flagellar apparatus. As shown in Fig. 1, both flagellar mutants were drastically (1,000-fold) and equivalently reduced in their abilities to enter cultured intestinal epithelial cells despite the application of a centrifugal force during the assay to facilitate bacteria-cell contact. These results are consistent with previous findings (17, 70, 75) and indicate that although motility *per se* is required for bacterial entry, internalization is not the result of events directly mediated by either flagellin or other proteins secreted via the flagellar apparatus.

Identification of Tn*552kan* insertion mutants of *C. jejuni* 81-176 defective for entry into cultured epithelial cells. Using the optimized invasion assay described in Materials and Methods, 3,200 Tn*552kan* transposon insertion mutants were screened for the ability to enter cultured epithelial cells. Since it had been previously shown (14, 17, 70, 75) and confirmed by our studies that bacterial motility is essential for invasion, a step was introduced into the screening protocol to identify nonmotile mutants prior to assaying them for epithelial cell invasion. This step identified 149 mutants (4.7% of the total number of mutants screened) that were nonmotile and therefore were not run through the epithelial cell invasion assay. Although the annotated genome sequence of *C. jejuni* NCTC 11168 lists 40 genes (2.4%) as being involved in the assembly and function of flagella (50), the actual number of genes that affect motility is thought to be significantly higher. In fact, comprehensive motility mutant searches in *E. coli* and *Bacillus subtilis* have demonstrated that mutations in ~4% of nonessential genes exhibit a motility phenotype (56). A random sampling of the nonmotile mutants isolated in the screen identified transposon insertions in *flhF*, *fliI*, *flgB*, *flgH*, *fliR*, *flgM*, *flgE*, and *pflA*, all of which encode components of the flagellar system (19, 75, 77; also data not shown). In addition, a number of mutants exhibited a wild-type motility phenotype when tested on motility plates but had a significant proportion of nonmotile bacteria when observed under the microscope. Although the molecular basis for this phenotype was not investigated, we hypothesize that the reduced motility may be due to either phase variation (6, 8, 22, 48) or reduced expression of genes encoding components of the flagellar system. Nevertheless, since motility plays a major role in the ability of *C. jejuni* to enter cultured epithelial cells, the presence of motility-defective mutants in the insertion pool that could not be eliminated by the motility plate assay required the examination of all noninvasive mutants under the microscope to identify those with possible motility defects. Whenever identified, these mutants were eliminated from the screen and were not considered further. Finally, a total of 52 insertion mutants were identified that retained wild-type motility in both plate and bacterial suspension assays but exhibited a reduced ability to invade cultured epithelial cells that ranged from 2- to 100-fold. The phenotype of these mutants was confirmed using a quantitative assay (see Materials and Methods), and the transposon insertion sites for each mutant were determined by nucleotide sequencing and are listed in Table 2.

Based on their predicted functions, the identified genes can be classified into the following groups.

(i) Resistance to sodium deoxycholate. The invasion assay protocol utilized in the screen includes a step in which the epithelial cells are lysed with sodium deoxycholate to release the internalized bacteria prior to their plating for enumeration. It has been previously reported that mutations in certain genes conferred increased sensitivity to bile salts, including sodium deoxycholate (41, 42, 57). Therefore, all mutants that exhibited an invasion phenotype were tested for their sensitivity to 0.1% sodium deoxycholate. Eleven mutants were identified that exhibited increase sensitivity to sodium deoxycholate. Retesting of these mutants with a modified invasion assay protocol that does not utilize sodium deoxycholate to lyse the epithelial cells determined that these mutants exhibit wild-type invasion levels (data not shown). Nucleotide sequencing established that all these mutants carry a transposon insertion within the *cmeABC* locus. These genes encode a multidrug efflux system that con-

TABLE 2. Location of some Tn*552kan* insertions in the *C. jejuni* 81-176 chromosome identified during the screen for noninvasive mutants

ORF (NCTC 11168/81-176)[a]	Insertion site/total length of ORF (nt)[b]	Annotation
Cj0039c/CJJ81176_0077	297/1,809 1080/1,809	*typA*, GTP-binding protein TypA
Cj0064c/CJJ81176_0102	1221/1,455	*flhF*, flagellar biosynthetic protein FlhF
Cj0081/CJJ81176_0118	453/1,563 1323/1,563	*cydA*, cytochrome *d* ubiquinol oxidase, subunit I
Cj0087/CJJ81176_0122	250/1,407	*aspA*, aspartate ammonia-lyase
Cj0140/CJJ81176_0176	50/867	Hypothetical protein
Cj0169/CJJ81176_0205	561/663	*sodB*, superoxide dismutase, Fe
Cj0190c/CJJ81176_0221	824/1,506	Mg chelatase-related protein
Cj0195/CJJ81176_0226	347/1,386	*fliI*, flagellum-specific ATP synthase FliI
Cj0227/CJJ81176_0252	625/1,182	*argD*, acetylornithine aminotransferase
Cj0261c/CJJ81176_0288	322/720	Conserved hypothetical protein
Cj0264c/CJJ81176_0291	2397/2,517	Biotin sulfoxide reductase
Cj0268c/CJJ81176_0295	1023/1,089 1049/1,089	SPFH domain/band 7 family protein
Cj0342c/CJJ81176_0366	562/2,826 1098/2,826 1438/2,826 1711/2,826	*uvrA*, excinuclease ABC, A subunit
Cj0365c/CJJ81176_0388	884/1,479 1479/1,779	*cmeC*, RND efflux system,[c] outer membrane lipoprotein CmeC
Cj0366c/CJJ81176_0389	963/3,123 1215/3,123 2098/3,123	*cmeB*, RND efflux system, inner membrane transporter CmeB
Cj0367c/CJJ81176_0390	1001/1,104	*cmeA*, RND efflux system, membrane fusion protein CmeA
Cj0411/CJJ81176_0435	1611/2,187	GTP-binding protein
Cj0454c/CJJ81176_0479	379/519 381/519	Hypothetical protein
Cj0456c/CJJ81176_0481	475/960 574/960 656/960	Hypothetical protein
Cj0528c/CJJ81176_0553	63/432	*flgB*, flagellar basal body rod protein FlgB
Cj0587/CJJ81176_0615	564/948	Membrane protein, putative
Cj0687c/CJJ81176_0710	64/699	*flgH*, flagellar L-ring protein FlgH
Cj0693c/CJJ81176_0716	126/933	*mraW*, *S*-adenosyl-methyltransferase MraW
Cj0762c/CJJ81176_0783	669/1,170	*aspB*, aspartate aminotransferase; *aspC*, aspartate aminotransferase
Cj0788/CJJ81176_0809	354/492	Conserved hypothetical protein
Cj0791c/CJJ81176_0812	152/1,269	Aminotransferase, putative
Cj0843c/CJJ81176_0859	1333/1,626	Soluble lytic murein transglycosylase, putative
Cj0924c/CJJ81176_0931	547/555	*cheB*, protein-glutamate methylesterase CheB
Cj1068/CJJ81176_1086	172/1,107 396/1,107	Membrane-associated zinc metalloprotease, putative
Cj1069/CJJ81176_1087	297/867	Conserved hypothetical protein
Cj1097/CJJ81176_1115	291/1,224	Sodium/dicarboxylate symporter
Cj1120c/CJJ81176_1138	636/1,773	*pglF*, general glycosylation pathway protein
Cj1161c/CJJ81176_1176	163/2,100 1895/2,100	Copper-translocating P-type ATPase
Cj1179c/CJJ81176_1194	647/768 649/768	*fliR*, flagellar biosynthetic protein FliR
Cj1198/CJJ81176_1213	31/495	*luxS*, autoinducer-2 production protein LuxS
Cj1209/CJJ81176_1223	735/1,554	HD/HDIG/KH domain protein
Cj1215/CJJ81176_1228	1061/1,161	Peptidase, M23/M37 family

Continued on following page

TABLE 2—*Continued*

ORF (NCTC 11168/81-176)[a]	Insertion site/total length of ORF (nt)[b]	Annotation
Cj1228c/CJJ81176_1242	293/1,419 663/1,419	*htrA*, protease DO
Cj1249/CJJ81176_1265	492/1,461 876/1,461 1140/1,461 1233/1,461	Hypothetical protein
CJJ81176_1314	657/897	Conserved hypothetical protein
Cj1341c/CJJ81176_1340	390/1,827 224/1,827 854/1,827	*maf6*, motility accessory factor/CJJ81176_1340 motility accessory factor
Cj1418c/CJJ81176_1417	1906/2,340 2028/2,340 2034/2,340	Part of *C. jejuni* capsule locus (Cj1413c - Cj1448c); conserved hypothetical protein
Cj1425c/CJJ81176_1424	410/1,020	Capsular biosynthesis sugar kinase, putative
Cj1428c/CJJ81176_1427	191/1,059 733/1,059	*fcl*, GDP-L-fucose synthetase
CJJ81176_1435	947/1,812 985/1,812	Putative sugar transferase
CJJ81176_1436	1816/2,181	Putative glycosyl transferase
Cj1464/CJJ81176_1457	48/198	*flgM*, hypothetical protein
Cj1540/CJJ81176_1525	106/810 454/810 711/810	Tungstate ABC transporter, periplasmic tungstate-binding protein, putative
Cj1565c/CJJ81176_1550	2065/2,367	*pflA*, paralyzed flagellar protein PflA
Cj1685c/CJJ81176_1677	72/837	*bioB*, biotin synthetase
Cj1729c/CJJ81176_0025	256/2,517	*flgE*, flagellar hook protein FlgE

[a] DNA sequences of *Tn552kan* insertions junction sites were used to determine the insertion site. The ORF designations correspond to the *C. jejuni* NCTC 11168 and 81-176 strains.
[b] Insertion site(s) within a given gene of *C. jejuni* 81-176 (The Institute for Genomic Research [TIGR] project identification number 16135; complete genome sequence). nt, nucleotide.
[c] RND, resistance-nodulation-cell division (efflux pump).

fers resistance to bile salts normally present in the intestinal tract of animals. Consequently, *cmeABC* mutants are defective for colonization in an animal model of *C. jejuni* infection (41, 42).

(ii) *C. jejuni* surface structures. A number of transposon insertions mapped to genes predicted to encode components or modifiers of putative surface structures. Among this group there were several insertions in genes involved in capsule polysaccharide biosynthesis and N-linked glycosylation, including Cj1414c, Cj1418c, Cj1422, Cj1425c, Cj1428c, Cj1440, and Cj1120c (*pglF*). Previous studies have shown that mutations in the capsule polysaccharide and N-linked glycosylation affected *C. jejuni* entry into cultured epithelial cells, and therefore these structures have been proposed to be directly involved in the adhesion and entry processes (1, 3, 31, 65). However, the modest defect in invasion observed in these mutants, which ranged from 2- to 3-fold, suggests that these structures may play only a secondary role in the invasion process, perhaps by promoting a more intimate interaction which may facilitate the stimulation of the entry event by other bacterial determinants.

(iii) Chemotaxis and other flagellum-associated loci. A number of mutants that showed a measurable defect in entry but retained full motility according to both the plate motility and microscopic observation assays were mapped to genes associated with the flagellar system. One of the transposon insertions inactivated the predicted homolog of CheB (Cj0924c), a putative methyl-accepting chemotaxis protein (MCP)-glutamate methylesterase, and exhibited a ~3-fold decrease in entry. We hypothesize that this entry defect may be due to the inability of this mutant strain, as well as other mutants of the *C. jejuni* chemotaxis system (23, 66, 76), to properly swim toward the cultured epithelial cells. Two insertions were mapped to *cj1340* and *cj1341c*, which belong to the *maf* (motility-associated factors) gene family. This gene family is prone to phase variation via a slipped-strand mispairing mechanism and is clustered in chromosomal regions encoding flagellar biosynthesis genes (32, 50). Consequently, they have been proposed to be involved in flagellar biosynthesis and/or flagellar phase variation although mutations in only one of the family members, *maf5*, were shown to result in a motility defect

(32). Although we could not detect a motility defect in the two *maf* mutant strains identified in this screen, we hypothesize that the rather minor (less than 3-fold) invasion defect displayed by these mutants may be due to a slight motility defect that cannot be detected with our assays.

(iv) General metabolism and housekeeping. A number of mutants mapped to genes involved in a variety of metabolic and housekeeping functions. Included in this group are genes involved in DNA repair (*cj0342c* [*uvrA*]), amino acid metabolism and transport (*cj0227* [*argD*] and *cj1097*, a putative transmembrane transport protein), purine biosynthesis (*cj1208*, a 5-formyltetrahydrofolate cyclo-ligase), RNA metabolism (*cj0153c*, an RNA methylase), oxidative stress (*cj0169* [*sodB*]), respiration (*cj0264*, a putative oxidoreductase, and *cj0081*, a cytochrome *bd* oxidase subunit), stress responses (*cj1228c* [*htrA*] and *cj0039c* [*typA*]), and carbon metabolism (*cj0087* [*aspA*] and *cj0762c* [*aspB*]). Another mutation mapped to Cj0190c, a homologue of Mg^{2+} chelatases, which catalyzes the ATP-dependent insertion of Mg into protoporphyrin IX and participate in the critical steps of (bacterio)chlorophyll (Bchl/Chl) synthesis (69). Most of the mutants in this category exhibited a rather minor invasion phenotype, and therefore their contribution to *C. jejuni* internalization was considered to be indirect. However, mutations in *aspA*, *aspB*, and *sodB* resulted in a rather strong phenotype (~50- to 100-fold decrease in invasion), and these genes therefore were chosen for further characterization (see below).

(v) Putative regulators. One of the mutants mapped to a gene that encodes a putative homolog of the regulator LuxS (*cj1198*), the autoinducer-2 (AI-2) synthase, suggesting that quorum sensing may affect the ability of *C. jejuni* to interact with host cells (9, 26).

(vi) Unknown function. Several insertions mapped to genes of unknown function, including the following: Cj0411, a putative ATP/GTP binding protein; Cj1209, an HD/KH domain-containing protein; Cj0261c, a putative *S*-adenosylmethionine (SAM)-dependent methyltransferase; Cj0268c, a putative transmembrane protein; Cj0791c, a putative aminotransferase; Cj0843c, a putative secreted transglycosylase; Cj1068, a putative peptidase M50 family protein; and Cj0454c, Cj0456c, and Cj0788. Interestingly, some of these proteins have been previously implicated in *C. jejuni* virulence. Cj0411, Cj0454c, and Cj0456c have been previously identified in various mutagenesis screens as required for efficient chicken colonization (16, 23). The rather modest defect in invasion exhibited by all of these mutants, however, suggests that the corresponding gene products may not be directly involved in mediating the invasion phenotype.

Comparison of the phenotype of the mutants isolated in this screen with previously identified invasion-defective mutants. Previous studies have identified a number of genes encoding factors putatively involved in mediating the ability of *C. jejuni* to enter cultured epithelial cells that did not affect motility. However, since these mutants were isolated in different bacterial strains and/or studied in different cell lines using different assay protocols, the comparison of those findings with findings reported here is difficult. Although some of those mutants were also identified in this screen (see above), a number of them were not, despite of the fact that our screen should have, statistically, picked them up. Therefore, to compare the phenotypes of previously identified invasion-defective mutants with the phenotype of the mutants isolated in this study, we constructed a number of *C. jejuni* 81-176 derivative strains carrying mutations in genes previously reported to be involved in invasion. In particular, we targeted *peb1A*, *ciaB*, *jlpA*, and *cj0977* because these genes have been reported in previous studies to have the strongest phenotypes (15, 27, 35, 52, 58). We then assayed their invasion phenotypes using the protocols and cell lines described in this study.

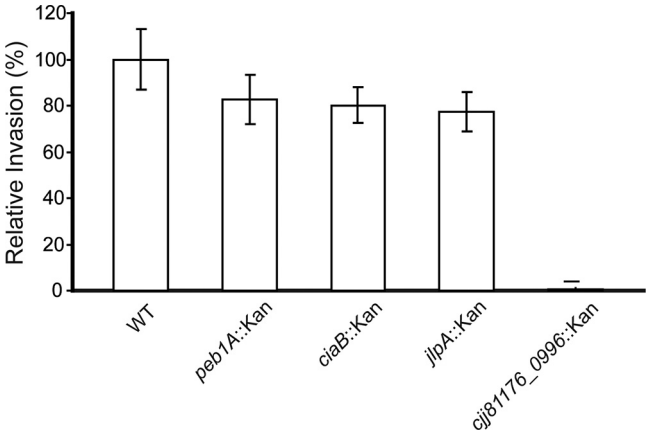

FIG. 2. Levels of internalization of previously reported invasion-defective mutants. T84 cells were infected with the wild-type *C. jejuni* 81-176 strain (WT) or the indicated isogenic mutants at an MOI of 10 for 2 h, followed by 3 h of incubation in the presence of gentamicin. A comparison of the levels of invasion is shown as the percentage of bacteria that survived treatment with gentamicin relative to that of the wild-type strain, which was set at 100%. The error bars represent the standard deviation of three independent determinations. The values of the *cjj81176_0996*::*kan* mutant strain were statistically significantly different ($P < 0.001$, Student *t* test) from those of the wild type. The values of the other mutant strains were not statistically significantly different from those of the wild type.

C. jejuni Peb1A is an aspartate/glutamate-binding protein of an ABC transporter that is essential for the uptake of dicarboxylic amino acids (40, 51). In addition, Peb1A has been proposed to be an important adhesion factor, and a loss-of-function mutation in this gene has been reported to reduce significantly *C. jejuni*'s ability to interact with epithelial cells or colonize experimental animals (52). However, we found that the introduction of an insertion mutation in this gene that resulted in a total loss of function did not affect the ability of *C. jejuni* 81-176 to attach and/or invade cultured epithelial cells under our standardized assay conditions (Fig. 2). These results are in disagreement with a previous report indicating a ~15-fold reduction in the levels of internalization into Henle 407 cells and a ~100-fold reduction in the levels of attachment to HeLa cells (52). We are not certain what the reasons for the discrepancy may be, but variations in the protocols, growth conditions, and/or cell lines used in the different studies may account for these differences.

Cultivation of *C. jejuni* in the presence of mammalian cells has been correlated with the *de novo* synthesis of a number of bacterial proteins as well as with an increase in bacterial invasion (34, 36, 58), suggesting that perhaps some of these *de novo* synthesized proteins may be involved in triggering the internalization process. One of these proteins, CiaB, has been sug-

gested to be necessary for entry since a null mutation in *ciaB* has been reported to cause a significant reduction in *C. jejuni* internalization into cultured cells (35, 58). We constructed a *ciaB*-deficient mutant of *C. jejuni* 81-176 and examined its ability to enter cultured intestinal epithelial cells. Surprisingly, we observed no measurable defect in the ability of this mutant to enter into cells (Fig. 2). The reasons for these discrepancies are unknown, but they may relate to differences in the strains or protocols used in the different studies. However, it should be pointed out that another laboratory also failed to confirm the phenotype of the *ciaB* mutant (15). In the original report, the *ciaB* mutant strain showing a defect in invasion was not complemented by reintroduction of a wild-type copy of the gene. Given the propensity of *C. jejuni* to undergo phase variation, it is possible that the phenotype originally ascribed to *ciaB* was due to an unlinked phenomenon.

JlpA is a surface-exposed lipoprotein in *C. jejuni* reported to play a role in mediating the adherence of this bacterium to host epithelial cells (27, 28). Although our screen did not identify a *jlpA* mutant, we investigated the potential contribution of JlpA to the *C. jejuni* invasion phenotype as assayed by our protocol. We constructed a *C. jejuni* 81-176 *jlpA* mutant strain and assayed the mutant for its ability to invade cultured epithelial cells. As shown in Fig. 2, the ability of the mutant strain to enter cultured epithelial cells was indistinguishable from that of the wild type.

A recent study reported that the gene *cj0977*, whose expression is coregulated with flagella, was required for *C. jejuni* virulence but not for motility (15, 54). Inactivation of *cj0977* resulted in a *C. jejuni* mutant that was motile but significantly (~3 logs) impaired in bacterial entry (15). We were surprised that a mutant with such a strong phenotype did not come up in our screen since the reported studies used the same *C. jejuni* strain (81-176) as we did. Furthermore, a 3-log defect would be hard to reconcile by slight differences between our assay and the protocol used in that study. We therefore constructed a loss-of-function mutant in *cj0977* and examined its ability to enter into cultured intestinal epithelial cells. We found that our *C. jejuni cj0977* mutant exhibits a drastic reduction in its ability to enter cultured intestinal epithelial cells (Fig. 2) (0.57% ± 0.31% of the wild-type levels of invasion in several repetitions of the experiment). These results therefore confirmed the reported invasion defect of this mutant. However, we observed that although this mutant scored as "motile" on standard motility plates, when resuspended in HBSS, the buffer used in the invasion assay, this mutant exhibited a significant motility defect, which was accentuated during the assay. We have found that this type of motility defect results in a drastic invasion defect, underscoring the central importance of motility in the internalization process. We therefore conclude that the invasion phenotype of the *cj0977* was due to its lack of robust motility and not to a direct role of the predicted protein in triggering the internalization event. The potential involvement of *cj0977* in regulating motility is entirely consistent with its structural similarity to FapR, a *B. subtilis* transcriptional regulatory protein, and its coregulated expression with flagellar genes (15).

Characterization of the invasion phenotype of *aspA*, *aspB*, and *sodB* mutants. Our transposon mutagenesis screen identified only three *C. jejuni* mutants that, while retaining wild-type motility, exhibit a significant (i.e., more than 20-fold) defect in the ability to enter nonphagocytic cells. These insertions mapped to *aspA*, *aspB*, and *sodB*. Because of their strong phenotype, these mutants were chosen for further characterization.

The *sodB* gene encodes a superoxide dismutase (SOD), whose primary function is to detoxify reactive oxygen intermediates generated during respiration and oxidative stress by catalyzing the conversion of superoxide (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (21, 46). SODs have been shown to be important virulence factors for a number of bacterial pathogens including *Campylobacter coli* and *C. jejuni* (53, 55). Previous studies have also reported an invasion defect for a *C. jejuni* strain carrying a loss-of-function mutation in *sodB* (53). However, this previous study did not distinguish whether this mutant had a defect in attachment, entry, or survival within cells. Defects in any of these phenotypes would have scored equally in the assay utilized in those studies. Therefore, we reexamined the phenotype of the *sodB* mutant in *C. jejuni* interactions with host cells. We first reconstructed the *sodB* mutant strain and examined its invasion phenotype by a gentamicin protection assay, as described in Materials and Methods. The reconstructed *sodB* mutant strain retained full motility and exhibited a significant invasion defect (Fig. 3A) (2.61% ± 0.76% of wild-type levels; three repetitions of the experiment), which is consistent with the findings of the previous study (53). Complementation of the mutant by reintroduction of a wild-type copy of *sodB* significantly restored its invasion phenotype (39.3% ± 3.0% of the wild-type levels; three repetitions of the experiment) (Fig. 3A), therefore confirming the linkage of the invasion phenotype to *sodB*. We next investigated whether the defect observed in the gentamicin protection assay was the result of a defect in entry, attachment, intracellular survival, or a combination of some or all of the above. Since the gentamicin protection assay cannot distinguish among these possibilities, we examined the ability of the *sodB* mutant to attach to cultured cells by enumerating all cell-associated bacteria after infection using CFU- and flow cytometry-based assays (see Materials and Methods). Both assays indicated that the *sodB* mutant exhibits a decreased ability to attach to cells that ranges between 6- and 7-fold (Fig. 3B and C). This defect, however, could not fully account for the larger defect observed with the gentamicin resistance assay, suggesting that the viability of the *sodB* mutant may decrease shortly after internalization. To address this issue, we examined the ability of the *C. jejuni sodB* mutant to enter cultured epithelial cells with a microscopy-based assay. This assay uses a staining protocol that can distinguish between internalized versus extracellular bacteria. Furthermore, this assay is independent of bacterial CFU recovery after infection. Using this assay, we detected a ~9-fold defect between in the levels of the internalized *sodB* mutant compared to the wild type (Fig. 3D), which is slightly larger than its defect in attachment. These results suggest a slight deficiency in the recovery of the *sodB* mutant shortly after infection, which, in combination with the attachment phenotype, may account for the bulk of the differences seen with the gentamicin assay. In fact, longer infection periods further accentuated the difference in intracellular survival rates between the wild-type and the Δ*sodB* mutant (Fig. 3E). Taken together, these results suggest that the reduced

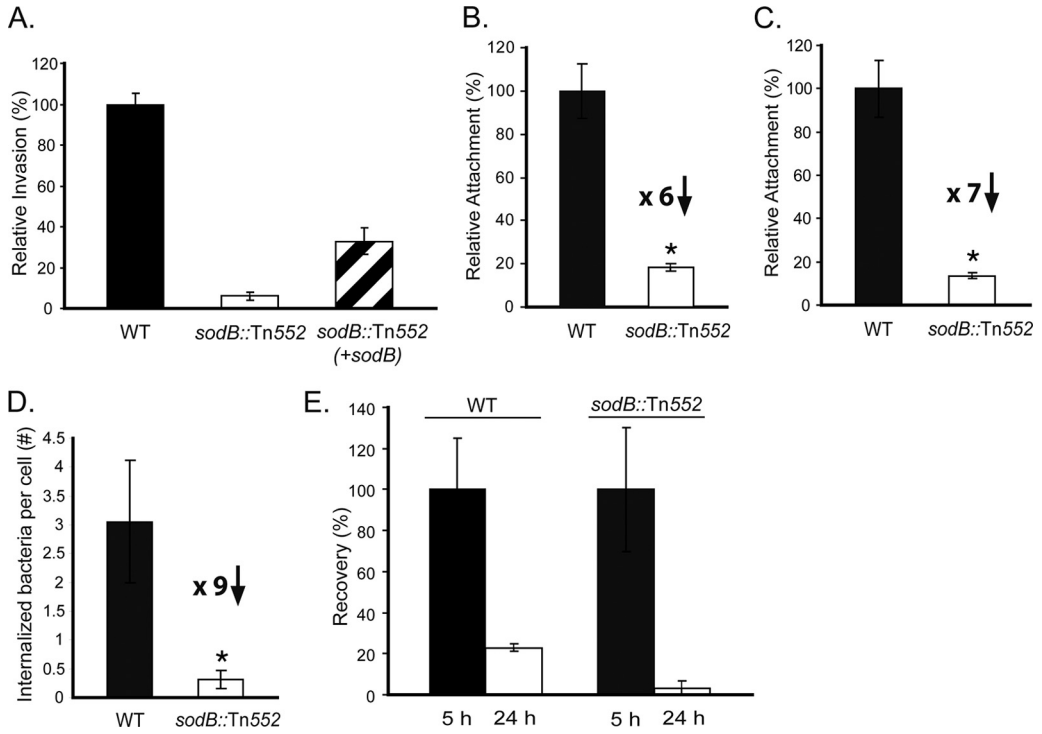

FIG. 3. Ability of *C. jejuni sodB* mutant to adhere, invade, and survive within cultured cells. (A) The *sodB* mutant is defective for entry into cultured cells. T84 cells were infected with the wild-type *C. jejuni* 81-176 strain (WT), the *sodB*::Tn*552* mutant derivative, or the complemented mutant (as indicated) at an MOI of 10 for 2 h, followed by a 3-h incubation in the presence of gentamicin. A comparison of the levels of invasion is shown as the percentage of bacteria that survived treatment with gentamicin relative to that of the wild-type strain, which was set at 100%. The error bars represent the standard deviation of three independent determinations. The values of the *sodB*::Tn*552* mutant strain were statistically significantly different ($P < 0.0001$, Student *t* test) from those of the wild type. (B, C, and D) The *sodB* mutant is defective for attachment to cultured cells. COS-7 cells were infected with *C. jejuni* 81-176, its *sodB*::Tn*552* mutant derivative, or the complemented mutant (as indicated) at an MOI of 50 for 2 h. Infected cell monolayers were washed twice and processed according to the indicated protocols as described in Materials and Methods and as follows: cells were lysed with 0.1% SOD to release intracellular bacteria and plated onto blood agar plates (B); cells were lysed, and the total bacteria were stained using a cell viability kit (BD Biosciences, San Jose, CA) and enumerated by flow cytometry (C); cells were fixed and processed with the primary (anti-*C. jejuni* 81-176) and secondary (Alexa Fluor-488 and Alexa Fluor-568) antibodies to enumerate extracellular and intracellular bacteria by immunofluorescence microscopy (D). Values indicate the levels of adhesion/invasion shown as a relative percentage of the wild type, which was considered 100%. The fold decrease in the *sodB* mutant's ability to attach to cells (B and C) and in the number of the mutant bacteria internalized (D) is indicated by the down arrows on the figure. The error bars represent the standard deviations of results for three independent determinations. *, statistically significant ($P \leq 0.005$, Student *t* test) different values from those of the wild type. (E) The *sodB* mutant is defective for intracellular survival. T84 cells were infected with the wild-type *C. jejuni* 81-176 strain (WT) or the *sodB*::Tn*552* mutant derivative (as indicated) at an MOI of 10 for 2 h, followed by 3 and 22 h of incubation in the presence of gentamicin. For each strain, levels of intracellular bacteria at 24 h are shown relative to the levels obtained at 5 h after infection, which was set at 100%. The error bars represent the standard deviation of three independent determinations. The survival values at 24 h of the *sodB*::Tn*552* mutant strain were statistically significantly different ($P < 0.005$, Student *t* test) from those of the wild type.

"invasion" phenotype of the *sodB* mutant observed using the gentamicin protection assay is not due to an invasion defect *per se*, as previously suggested (53), but to a combined defect in bacterial adhesion and impaired viability inside the cell.

The *aspA* and *aspB* genes encode aspartate ammonia-lyase and aspartate aminotransferase, respectively. The activity of these enzymes leads to the production of fumarate, which constitutes both a carbon source as well as an alternative electron acceptor during anaerobic respiration (61, 62). Given the function of these genes, it was surprising to observe such a strong invasion phenotype. We first tested the susceptibility of these mutants to the concentration of sodium deoxycholate used in our invasion assay. Exposure of either mutant to 0.1% sodium deoxycholate did not alter their plating efficiencies (data not shown). To confirm that the invasion phenotype was associated with the inactivation of *aspA* or *aspB*, a wild-type copy of these genes was introduced into the respective strains, which were then tested for their invasion phenotypes. Both mutants were fully complemented by the introduction of wild-type copies of the respective genes, confirming that the invasion phenotype observed was associated with the insertional inactivation of either *aspA* or *aspB* and not due to potential polar effects on downstream genes (Fig. 4A and B). We then tested the growth characteristics of the *aspA* and *aspB* mutants in rich medium. Both strains initially grew to levels similar to those of the wild type, but their growth plateaued when they reached an OD_{600} of ~0.5. However, the wild-type level of growth was recovered upon the addition of 20 mM fumarate (Fig. 4C). We then tested the invasive ability of the mutants during their growth cycle. We found that early during growth, the invasive ability of the *aspA* or *aspB* mutant was only ~4-fold lower than that of the wild type.

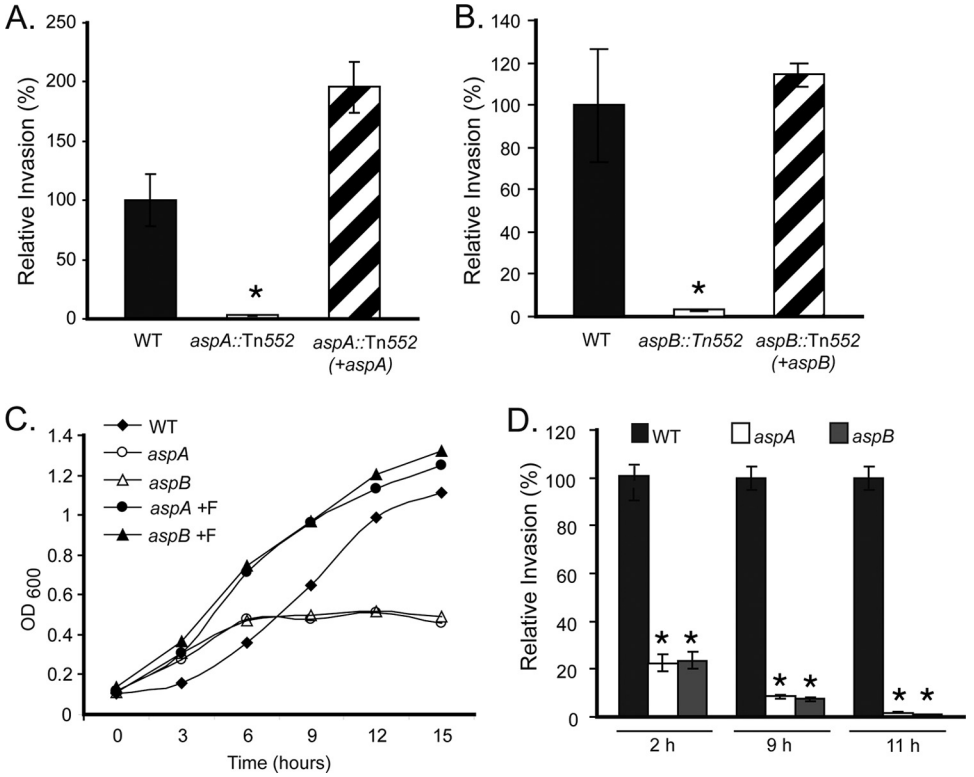

FIG. 4. Ability of *C. jejuni aspA* and *aspB* mutants to invade cultured cells. (A and B) T84 cells were infected with wild-type *C. jejuni* 81-176 (WT), its isogenic *aspA*::Tn*552* or *aspB*::Tn*552* mutant, or the complemented mutant strains, as indicated. Levels of bacterial internalization are shown as the percentage of bacteria that survived treatment with gentamicin relative to that of the wild-type strain, which was set at 100%. The error bars represent the standard deviation of three independent determinations. *, values were statistically significantly different ($P < 0.0001$, Student *t* test) from those of the wild type. (C) Growth characteristics of the *aspA* and *aspB* mutants. Growth curves of the wild-type *C. jejuni* 81-176 and its isogenic *aspA*::Tn*552* or *aspB*::Tn*552* mutant grown in BHI medium with or without the addition of fumarate (F) (20 mM). (D) Invasion ability of the *aspA* and *aspB* mutants during growth. T84 cells were infected with wild-type *C. jejuni* 81-176 (WT) or its isogenic *aspA*::Tn*552* or *aspB*::Tn*552* mutant which had been grown in BHI medium for the indicated times. Levels of bacterial internalization are shown as the percentage of bacteria that survived treatment with gentamicin relative to that of the wild-type strain, which was set at 100%. The error bars represent the standard deviation of three independent determinations. *, values were statistically significantly different ($P < 0.0001$) from those of the wild type.

However, as the mutants continued to grow, their invasive ability progressively decreased, and at the late stationary phase of growth, the invasive abilities of both mutants sharply declined (Fig. 4D). The fact that these mutants were able to enter cultured intestinal cells during the early growth phase clearly indicated that neither AspA nor AspB is directly involved in mediating entry. Rather, these results suggest that absence of AspA or AspB translates into undetermined physiological changes that render these mutants unable to enter cultured cells. Consistent with this hypothesis, the invasion phenotype could be recovered by the addition of fumarate, which also recovered the growth characteristics of the mutants (Fig. 4C; see also Fig. S2 in the supplemental material).

***aspA*, *aspB*, and *sodB* mutants are defective in mouse colonization.** To further evaluate the potential role of AspA, AspB, and SodB in virulence, we examined the ability of mutant strains to colonize animals using a recently developed mouse model of infection. A *C. jejuni aspA*, *aspB*, or *sodB* mutant strain was administered intraperitoneally simultaneously with the wild type or a complemented derivative to MyD88-deficient mice, and the presence of the different strains in the feces of infected animals was monitored over time, as described in Materials and Methods. Five out of seven mice cleared the *aspA* mutant by the 10th week after infection, meaning that this strain could not be detected in the feces of infected animals (Fig. 5A). Consistent with a defect in colonization, the *aspA* mutant was significantly reduced in the tissues of infected animals 10 weeks after infection (Fig. 5B). Similar results were obtained with the *aspB* mutant, and reduced or undetectable CFU counts of the mutant were recovered in both the feces and tissues of infected animals when the mutant was administered simultaneously with the wild type (Fig. 5C and D).

The *C. jejuni sodB* strain could not be detected in the feces of four out of five mice as early as 2 days postinfection. One mouse still shed a small number of the *sodB* strain cells 1 week later (2×10^3) while the levels of the complemented strain remained high (5×10^5) in all animals throughout the experiment (Fig. 6A). Two weeks after infection, all animals were sacrificed, and their tissues were examined for the presence of the *sodB* mutant and complemented strain. Although the complemented strain was recovered from the intestine, liver, and spleen of all mice, the *sodB* mutant was not present in any of the tissues examined (Fig. 6B). Taken together, these results

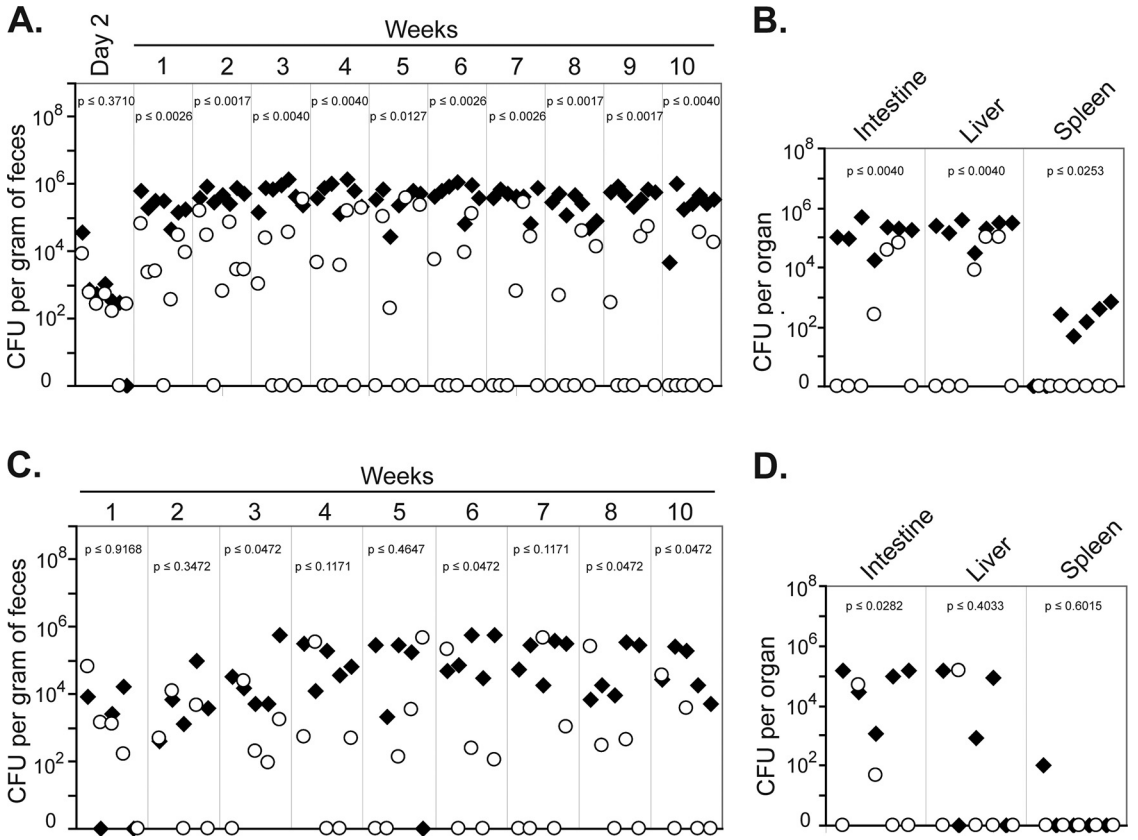

FIG. 5. *C. jejuni aspA* and *aspB* mutants are attenuated in a mouse colonization model. Wild-type *C. jejuni* and its *aspA* (A and B) or *aspB* (C and D) mutant derivative were simultaneously administered intraperitoneally to 8-week-old MyD88- and Nramp1-deficient mice. The numbers of CFU for the two competing strains in the feces of mice were enumerated by differential plating at the indicated times (A and C). At the end of the experiment mice were sacrificed, and their internal organs were homogenized and plated on differential medium (B and D). Each symbol represents the number of CFU of an individual mouse. ◆, *C. jejuni* wild-type strain; ○, *aspA* or *aspB* mutant strain.

indicate that AspA, AspB, and SodB are required for *C. jejuni* host colonization.

DISCUSSION

We have conducted a transposon mutagenesis screen to identify *C. jejuni* genes directly involved in the cellular internalization process. The screen was designed to eliminate non-motile mutants since it is well established that motility is essential for *C. jejuni* entry. Although it is difficult to determine whether our screen was exhaustive, the fact that many non-identical insertions in the same genes were often obtained suggests that the screen most likely approached saturation. With the exception of insertions in three genes (*aspA*, *aspB*, and *sodB*), our screen did not identify mutations that were

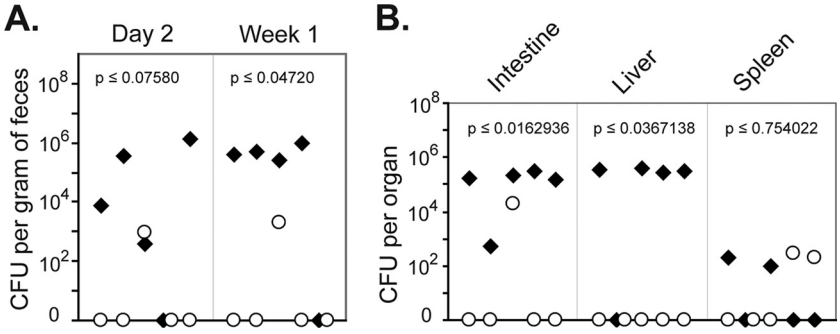

FIG. 6. *C. jejuni sodB* is attenuated in a mouse colonization model. *C. jejuni sodB* and its complemented derivative were simultaneously administered intraperitoneally to 8-week-old MyD88- and Nramp1-deficient mice. The numbers of CFU for the two competing strains in the feces of mice were enumerated by differential plating at the indicated times (A). At the end of the experiment mice were sacrificed, and their internal organs were homogenized and plated on differential medium (B). Each symbol represents the number of CFU of an individual mouse. ○, *C. jejuni sodB* mutant; ◆, complemented mutant.

impaired in entry to a large extent (more than 10-fold) although it identified numerous mutations that exhibited a modest (less than 5-fold) phenotype. We hypothesize that the modest phenotypes associated with the rest of the mutants suggest that the identified genes do not encode factors directly involved in bacterial entry.

Our screen identified mutations in *aspA*, *aspB*, and *sodB* that demonstrated a rather strong invasion phenotype. The phenotype of the *aspA* and *aspB* mutants could be reversed by either altering the growth conditions or supplying the strain with fumarate, the product of the enzymatic reactions associated with these gene products. These observations led us to conclude that AspA and AspB affect the invasion phenotype indirectly, perhaps by indirectly influencing the envelope composition of the bacteria under certain growth conditions and, hence, its invasiveness. Further studies of these phenotypes may yield information relevant to the understanding of the mechanisms *C. jejuni* internalization into host cells.

The defect in attachment and entry of the *sodB* mutant is intriguing although we do not favor a direct role of SodB in the entry process. While the reduced intracellular survival of the *sodB* mutant could be due to an increased sensitivity to reactive oxygen species that *C. jejuni* may be exposed to within the intracellular vacuole, its defect in attachment is more difficult to explain. Reports have shown that in *E. coli*, a *sodB* mutant constitutively expresses a set of outer membrane proteins, which are normally repressed in the wild type (43, 44). It is therefore possible that an equivalent remodeling of the outer membrane in the *C. jejuni sodB* mutant may alter the availability of putative envelope proteins directly involved in mediating cellular attachment. More experiments will be required to clarify these issues.

Since our studies also indicated that AspA, AspB, and SodB do not play a direct role in entry, it follows that our screen most likely did not identify "true" invasion determinants. Some previous studies have identified mutations that retained wild-type motility but exhibited rather drastic invasion phenotypes. Since those mutations did not come up in our screen, we constructed strains carrying mutations in these factors and compared their abilities to enter cells. Our studies could not confirm the phenotypes of three of these mutants, *jlpA*, *ciaB*, and *peb1A* (27, 35, 52), indicating that at least in *C. jejuni* 81-176, these genes played no measurable role in bacterial entry when assayed under the conditions used in this study. We confirmed the reported phenotype of a mutation in *cj0977* (15) and observed a rather severe invasion defect. However, our data indicate that although this mutant does not show a significant motility defect when assayed on motility plates, it does show a rather strong motility defect when assayed in liquid. This observation is consistent with the reported coregulation of expression of *cj0977* with other motility genes and also explains why this mutant did not come up in our screen since we eliminated from considerations all motility-defective mutants. Given the demonstrated importance of motility in cell invasion, the phenotype of this mutant is likely due to a role of Cj0977 in motility rather than to direct involvement in the entry event. In fact, Cj0977 is a homolog of FapR, a transcriptional regulatory protein of *B. subtilis*, suggesting that it may be involved in regulating flagellar gene expression. This observation also indicates that motility plates may not be a reliable indicator of the true motility phenotype of a given mutant since more subtle defects in motility may escape detection by this method.

Assuming that mutations in genes directly involved in entry should result in strong phenotypes, the question, then, is why did this rather extensive screen fail to identify those genes? There are a number of possibilities. First, although extensive, it is possible that our screen simply missed these mutants. We find this possibility unlikely, considering the large number of mutants screened and the relatively small size of the *C. jejuni* genome, particularly considering the fact that essential genes would not be targets of the screen. Indeed, different insertions in many genes were repeatedly obtained, which further supports the notion that our screen approached saturation. Second, it is possible that elimination of nonmotile genes resulted in the elimination of mutations in putative invasion genes. For example, since the transposon insertions obtained in this screen are polar, insertions in invasion genes located in the same operon as a downstream motility gene would not be recovered in this screen. Another possibility is that genes responsible for *C. jejuni* invasion or located in the same operon could be essential for bacterial viability and therefore would not be identifiable with the approach utilized in this study. Finally, the invasion determinants may be redundant, and therefore inactivation of a single invasion gene would not lead to an invasion phenotype.

Our results also highlight the challenges associated with the identification of true invasion genes. Many of the mutants we identified as invasion-defective based on the standard gentamicin protection assay turned out to have other defects that were not related to entry. Only after careful examination with alternative assays were we able to clarify the true phenotypes of those mutants. It is possible that some of these challenges may explain our inability to confirm previous invasion phenotypes of a number of mutants reported to exhibit internalization defects.

This study also highlights the close relationship between *C. jejuni* physiology and metabolism and its ability to enter and survive within cells. For example, our screen identified mutations in *aspA* and *aspB*, which showed a drastic phenotype in our assay. However, subsequent analysis indicated that the phenotype was highly dependent on the *C. jejuni* growth phase.

In conclusion, although our screen identified a number of genes that are important for *C. jejuni* colonization, the identification of *C. jejuni* determinants directly involved in entry remains elusive.

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